General and Comparative Endocrinology 282 (2019) 113205

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Screening for *Dmrt* genes from embryo to mature *Macrobrachium rosenbergii* prawns

Faiza Amterat Abu Abayed[a], Rivka Manor[a,b], Eliahu D. Aflalo[a], Amir Sagi[a,b,*]

[a] Department of Life Sciences, Ben-Gurion University of the Negev, P.O.B. 653, Beer-5 Sheva 84105, Israel
[b] National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, P.O.B. 653, Beer-Sheva 84105, Israel

ARTICLE INFO

Keywords:
Crustacea
Dmrt
Embryo
IAG
Larva and post larvae transcriptomic libraries
Macrobrachium *rosenbergii*
Transactivation domain (TAD)
Zinc finger

ABSTRACT

The doublesex and mab-3 related transcription factor (Dmrt) gene family is known to be related to the sexual regulators doublesex of arthropods and mab-3 of annelids and to hold highly conserved functions in sexual determination and differentiation across phyla. Here, we report a study of the Dmrt gene family in the freshwater prawn Macrobrachium rosenbergii, a crustacean whose sexual differentiation has been widely researched. A wide transcriptomic screen, from the embryo to the adult *M. rosenbergii*, identified five novel *Dmrt* genes (*MroDmrts*) and confirmed two known *MroDmrts*. The seven *MroDmrts* encode proteins of 275–855 amino acids; each protein contained at least one conserved DNA-binding DM domain, which is typical of Dmrt proteins, and five proteins contained 1–4 transactivation domains (TADs). Importantly, in the embryonic, larval and post-larval stages, *MroDmrt* genes exhibited time-dependent expression patterns rather than sex-specific expression. *In-silico* screening of the expression of the *MroDmrt* genes in adult males revealed the enrichment of *MroiDmrt1b* and *MroiDmrt1c* in the androgenic gland (AG) as compared to the eyestalks. *In vivo* silencing of the androgenic gland insulin-like (*IAG*) encoding gene significantly decreased the expression of the above two *Dmrt* genes, while not affecting the expression of control genes, thereby suggesting the possible role of these two genes in the IAG-switch and in sex-differentiation processes.

1. Introduction

The freshwater prawn *Macrobrachium rosenbergii* is one of the best investigated crustacean species, with studies ranging from its aquacultural importance (Nair et al., 2006; New, 2008); through its typical social structure (Karplus and Sagi, 2000; Kuris et al., 1987) and physiology (Chen et al., 2003; Manush et al., 2004; Zhu et al., 2018), to its sexual differentiation and reproductive biology on the protein (Okumura and Hara, 2004), transcriptomic (Jung et al., 2016; Sharabi et al., 2016; Shpak et al., 2017) and genomic levels. In the last regard, a high-quality *M. rosenbergii* genome was recently sequenced (Levy, personal communication), revealing sex-specific chromosomal regions. This prawn is thus an ideal species for the study of the important doublesex and mab-3 related transcription factor (Dmrt) gene family; these genes are related to the sexual regulators doublesex (*dsx*) of arthropods and *male-abnormal-3* (mab-3) of annelids and hold highly conserved functions in sexual determination and differentiation across phyla (Roth et al., 2013; Veenstra, 2016).

The *Dmrt* gene family encodes putative transcription factors that contain a common zinc finger DNA binding motif known as the DM domain. The transcriptional activity of such a Dmrt protein is determined by its C terminus through its transactivation domain/s (TAD/s) (Ma et al., 2014; Mapp and Ansari, 2007). Some Dmrt proteins are known to be involved in sex determination and/or sex differentiation (Raymond et al., 1998) in insects (Miller et al., 2003), nematodes and vertebrates (Kopp, 2012), but functional information regarding other gene family members and other phyla is limited. For example, the *dsx* gene of the insect *Drosophila melanogaster* was the first family member to be discovered in the sex determination cascade (Hildreth, 1965). In the nematode *Caenorhabditis elegans*, it has been shown that the DM domain gene *male abnormal-3* (*mab-3*) is required for male-specific development (Hodgkin, 2002). In vertebrates, orthologs of *Dmrt* genes, e.g., human *Dmrt1*, *Dmrt2*, and *Dmrt3*, have been identified and shown to be involved in sexual development (Raymond et al., 1999). However, it is only recently that scientists have begun to study *Dmrt* genes in crustaceans. The first crustacean DM-domain gene to be reported was the *EsDmrt-like* gene – identified in the Chinese mitten crab *Eriocheir sinensis* – which presents a testis-specific expression pattern (Zhang and Qiu, 2010). Other *Dmrt* genes were subsequently reported in a variety of crustacean species; e.g., in *Daphnia magna* the functional *Dmrt* gene,

* Corresponding author at: National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, P.O.B. 653, Beer-Sheva 84105, Israel.
E-mail address: sagia@bgu.ac.il (A. Sagi).

https://doi.org/10.1016/j.ygcen.2019.06.009
Received 30 January 2019; Received in revised form 28 May 2019; Accepted 11 June 2019
Available online 12 June 2019

DmaDsx1, was found to exhibit a sexually dimorphic expression pattern and to be responsible for male-specific trait development (Kato et al., 2011). The first heterogametic sex-linked *Dmrt* gene, *Sv-iDMY*, to be discovered in an invertebrate species was found in the Eastern spiny lobster, *Sagmariasus verreauxi* (Chandler et al., 2017). In *M. rosenbergii*, two *Dmrt* genes, designated *MroDmrt11E* and *MroDmrt99B*, have been identified (Yu et al., 2014). It has been shown that *MroDmrt11E* RNAi induced a significant decrease of the transcript of the androgenic gland (AG) insulin-like hormone encoding gene (*IAG*), an important regulatory gene in the male determination mechanism of the prawn, while silencing of *MroDmrt99B* had no effect on genes related to sexual development (Yu et al., 2014).

The AG has long been recognized as an endocrine organ unique to male crustaceans (Charniaux-Cotton, 1954), playing a vital role in sexual differentiation, including the development of primary and secondary sexual characteristics (Khalaila et al., 2001; Sagi and Cohen, 1990; Sagi et al., 1990). In *M. rosenbergii*, ablation of the AG at an early stage of development results in sex reversal of male to female (Sagi et al., 1990). Conversely, AG implantation into immature females inhibits vitellogenesis and results in functional sex reversal of female to male (Levy et al., 2016; Malecha et al., 1992). It has been hypothesized that the IAG induces the activity of male differentiation genes and represses genes responsible for female differentiation. Indeed, silencing of *M. rosenbergii IAG* (*Mr-IAG*) at early post- larval stages caused a full and functional sex reversal of males to neo-females (Ventura et al., 2012); thus, this process was termed the IAG-switch (Levy et al., 2016) between maleness and femaleness. However, the causative relationship between the IAG-switch and members of the *Dmrt* gene family remains to be elucidated.

In this study on *M. rosenbergii*, we report the identification of novel *Dmrt* genes, termed *MroDmrts*, together with their characterization and *in-silico* temporal expression in male and female embryos, larvae, and post-larvae, and in the AGs and eyestalks of male adults. The study of the *MroDmrt* genes revealed enrichment of two *MroDmrt* genes in the AG and their possible role in IAG-switch.

2. Materials and methods

2.1. Identification of putative DMRT family genes

Genes encoding putative Dmrt proteins were mined from a large array of *M. rosenbergii* transcriptomic libraries (Sharabi et al., 2016), either by a key-word-based search or by using *Daphnia magna* DMRT protein sequences (accessions: KZS07196.1, BAG12873.1, BAG12872.1, BAG12871.1 and BAJ78309.1) as a query. The searches yielded several sequences, which then served as queries in BLAST searches designed to reveal the similarity between our hypothetical sequences and the homologs in the NCBI database. The complete transcripts were obtained (for all genes excluding *MroDSX* due to its low level of expression) using rapid amplification of cDNA ends (RACE), performed with the Clontech SMART RACE kit (BD Biosciences, Palo Alto, CA), according to the manufacturer's instructions, and validated by Sanger sequencing. To obtain the deduced protein sequences, full-length cDNA of *MroDmrts* was computationally translated using the ExPASy Proteomics Server (http://web.expasy.org/translate/), and the most likely (i.e., the longest) frame was selected. Conserved domains were identified in the putative MroDmrt proteins using the Simple Modular Architecture Research Tool (SMART) (Schultz et al., 1998). In addition, TAD prediction was performed using the Nine Amino Acids Transactivation Domain 9aaTAD Prediction Tool (http://www.med.muni.cz/9aaTAD/) (Mapp and Ansari, 2007). To further characterize the above Dmrt proteins, a few homologous proteins from crustaceans, other arthropods and vertebrates were selected for phylogenic analysis by ClustalW alignment (Larkin et al., 2007) using the default parameters. The proteins used to construct the tree are given in Table 1. The neighbor-joining phylogenetic analysis was conducted with MEGA 7 (Kumar et al., 2016) with default parameters. Nematode *CelMAsB3* served as an outgroup.

2.2. Embryo transcriptomic library

All-female (Levy et al., 2016) and all-male (Ventura et al., 2012) *M. rosenbergii* progenies were produced as we have described previously. Small amounts of eggs containing developing embryos were sampled using forceps from egg berried females bearing either female or male embryos on days 1, 3, 5, 11 and 17 in 3 replicates per day (a total of 30 samples). Total RNA was extracted with the EZ-RNA Total RNA Isolation Kit (Biological Industries) according to the manufacturer's instructions. RNA samples were sequenced using Illumina Technology, yielding paired-end 100-bp reads. Subsequent bioinformatic analyses were performed at the Bioinformatics Core Facility of The National Institute for Biotechnology in the Negev (NIBN), using the NeatSeqFlow workflow platform (Sklarz et al., 2017) and R/Bioconductor. To create a comprehensive reference transcriptome, we examined previous RNA-Seq datasets (Table 2) produced by our group and our collaborators as well as publicly available datasets for inclusion in the transcriptome assembly. Reads from each of the samples of the current study were aligned to the reference transcriptome and quantified. Raw read counts (average of 13.2 ± 3.3 million read pairs per sample) were submitted to DESeq2. Counts were normalized using the Variance Stabilizing Transformation (VST). A BLAST search was performed using *MroDmrt* gene sequences as a query. To find the expression of *MroDmrt*s in the embryonic stages, the matched contigs were used for clustering according to their expression patterns in terms of sex and time.

2.3. Spatiotemporal expression of Dmrts

Sequence reads from male and female larvae, from male and female post larvae (PL) (Ventura et al., 2013) and from the AGs and eyestalks of three different male morphotypes – blue claw (BC), orange claw (OC) and small male (SM) (Sharabi et al., 2016) – were mapped to the *MroDmrt* reference sequences using the CLC Genomic Workbench7.3 (CLC Bio; default parameters). The numbers of *MroDmrt*-mapped reads per sample were normalized as described before (Sharabi et al., 2016). In addition, total RNA was extracted from the AGs, testes and ovaries using the EZ-RNA Total RNA Isolation Kit (Biological Industries, Beit Haemek, Israel) according to the manufacturer's instructions. cDNA was synthesized in a reverse-transcriptase reaction containing 1 μg of total RNA by using a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's instructions. Real time RT-PCR was conducted to measure *MroiDmrt1a, MroiDmrt1b, MroiDmrt1c and MroDmrt11E* expression levels in ovary, testis and AG, using specific primers and probes from the Universal Probe Library (Roche) (Table 3). For normalization, *Mr-18S* (GenBank accession no.GQ131934) was used with specific primers as described in Ventura et al. (Ventura et al., 2012) and Universal Probe Library Probe 152 (Roche), with the SensiFAST Probe Hi-ROX Mix (BIOLINE). Reactions were performed with the ABI Prism 7300 Sequence Detection System (Applied Biosystems).

2.4. Effects of Mr-IAG silencing on expression of Dmrt genes

To further investigate possible involvement of *MroDmrt* genes in the *Mr-IAG*-switch pathway, the two *MroDmrt* genes that were found to be highly expressed in the AG (*MroiDmrt1b* and *MroiDmrt1c*) – compared to eyestalks – were further studied. The expression levels of those two genes were quantified by qPCR in prawns that had been injected with the entire *Mr-IAG* dsRNA (ds*Mr-IAG*) open reading frame (ORF) (n = 8), as previously described by Shpak et al. (2017), and in a non-injected group (n = 7) as a negative control. In this experiment, small *M. rosenbergii* males (mean body weight of 11.6 g) were injected once with 5 μg of ds*Mr-IAG*/g body weight, and then two days post-injection, the control and injected males were anesthetized in ice cold water and

Table 1
Proteins used for the construction of the phylogenetic tree.

Genus and species	Proteins
M. rosenbergii	doublesex and mab-3 related transcription factor 99B, AHI47025.1 and doublesex and mab-3 related transcription factor 11E, AHI47024.1
Aedes aegypti	doublesex- and mab-3-related transcription factor A2, XP_001649612, male specific isoform 1 doublesex, ACY78701
Anopheles gambiae str. PEST	AGAP001388-PA, XP_321748, AGAP000431-PA, XP_310668, AGAP004050-PC and XP_560052
Apis mellifera	doublesex isoform M, NP_001104725.1
Bombyx mori	doublesex isoform M, NP_001104815.1
Caenorhabditis elegans	protein male abnormal 3, NP_001022464
Danio rerio	doublesex- and mab-3-related transcription factor 1 isoform 1, NP_991191.2, doublesex- and mab-3-related transcription factor 2, NP_571027.1, doublesex- and mab-3-related transcription factor 3a, NP_001005779.2, doublesex- and mab-3-related transcription factor 5, AAU85258.1
Daphnia magna	doublesex-mab related 93B, BAG12872, doublesex-Mab related 99B, BAG12873.1, doublesex-mab related 11E, BAG12871, doublesex1-alpha BAJ78307.1, doublesex2 BAJ78309.1
Drosophila melanogaster	doublesex-Mab related 11E, NP_511146.2, doublesex-Mab related 93B, NP_524428.1, doublesex-mab related 99B, NP_524549.1, doublesex, isoform A, NP_731197.1
Eriocheir sinensis	Dmrt-like protein, ADH15934.1
Fenneropenaeus chinensis	doublesex, AUT13216.1
Gallus gallus	doublesex- and mab-3-related transcription factor 1, Q9PTQ7, doublesex- and mab-3-related transcription factor 2, XP_003643035, doublesex- and mab-3-related transcription factor 3, XP_429193.2, doublesex- and mab-3-related transcription factor B1, NP_001232910
Homo sapiens	doublesex- and mab-3-related transcription factor 1 isoform 1, NP_068770.2, doublesex- and mab-3-related transcription factor 2 isoform 1, NP_006548.1, doublesex- and mab-3-related transcription factor 3, NP_067063.1, doublesex- and mab-3-related transcription factor A1, NP_071443.2, doublesex- and mab-3-related transcription factor A2, NP_115486.1, doublesex- and mab-3-related transcription factor B1, NP_149056.1, doublesex- and mab-3-related transcription factor C2, NP_001035373.1
Mus musculus	doublesex- and mab-3-related transcription factor 1, doublesex- and mab-3-related transcription factor 1, NP_056641.2, doublesex- and mab-3-related transcription factor 2, NP_665830.1, doublesex and mab-3 related transcription factor 3, AAN77230.1, doublesex- and mab-3-related transcription factor A1, NP_783578.1, doublesex- and mab-3-related transcription factor 5, AAN10254, mCG16080, EDL30774, mCG16080, EDL30774
Oryzias latipes	doublesex- and mab-3-related transcription factor 1Y, NP_001098150.2, DMRT2, AAL02163.1, OlaDMRT4, BAB63259.1, doublesex and mab-3 related transcription factor 5, BAD00703.1
Sagmariasus verreauxi	iDmrt1, ARK36620.1, iDMY, ARK36623.1, DSX, ARK36621.1, Dmrt11E, ARK36622.1
Takifugu rubripes	doublesex and mab-3 related transcription factor 1, NP_001033038, doublesex- and mab-3-related transcription factor 2, NP_001033035.1, doublesex- and mab-3-related transcription factor 3, NP_001033034.1, doublesex- and mab-3-related transcription factor A1, NP_001033037.1, doublesex- and mab-3-related transcription factor A2, NP_001033039.1
Xenopus laevis	doublesex- and mab-3-related transcription factor 1A, NP_001089969.1, doublesex and mab-3 related transcription factor 2 L homolog, NP_001089725.1, DMRT4, AAV66322.1, doublesex- and mab-3-related transcription factor A2, NP_001089148.1

Table 2
RNA-Seq datasets that were used for inclusion in the transcriptome assembly.

Dataset name or SRA accession	Description	No. of raw reads post QC
Current study and *M. rosenbergii* composite transcriptome	Embryos, larvae and post larvae (whole body), hepatopancreas, muscle, testis, eyestalk, claw, stomach and androgenic gland. Ben-Gurion University line *M. rosenbergii*	1,040,999,996
SRX092198	Gill, hepatopancreas and muscle tissues in *M. rosenbergii*	35,394,036
SRX097638		22,638,102
SRX097639		24,210,188
SRX2691440	Prawn antennal gland	65,076,128
SRX760284	Total RNA from eyestalks, CNS, and ovaries	37,925,076
SRX760285		37,925,078
SRX760286		37,925,402
SRX859032	Blastocyst	52,013,352
SRX608051	Blastocyst	54,708,014

Table 3
Primers used for qPCR quantifying the expression levels of *MroDmrt* genes and *Mr-actin* in the *Mr-IAG* silencing experiment.

	F primer	R primer	Probe
MroiDmrt1a	tacgagagcgagggcaag	ttcccgtggttcttgcag	38
MroiDmrt1b	gctgtgggattggaatcaa	tgatgaacaacgctgacctaa	22
MroiDmrt1c	aatttggagtacagcctgagga	tcagcatctatggtatttgtaggc	152
MroiDmrt1d	ggattcatcgacttccttcg	gaggcggataaggaggtgt	132
MroDmrt11E	aatgagcaatctccgtcagc	ttgtctttcgctcaagtaggg	113
Mr-actin	tcaggtcatcaccatcggta	gaaggatggctggaacagag	104

dissected. Total RNA was extracted from the AGs and cDNA was synthesized as described above. To validate the silencing of *Mr-IAG,* its relative expression levels were measured as described in (Ventura et al., 2012) with the above-mentioned mix from BIOLINE and Universal Probe Library Probe 144 (Roche). To quantify the relative expression of the genes designated *MroiDmrt1b* and *MroiDmrt1c* (see Section 3.1) in the above males, real time RT-PCR was done as described before. As controls, we tested the expression levels of *MroiDmrt1d* and *Mr-actin* to show the specific effects of *Mr-IAG* silencing, using specific primers and probes (Table 3).

3. Results

3.1. Identification of putative DMRT family genes

A search of a *M. rosenbergii* composite transcriptomic library (Sharabi et al., 2016) revealed seven transcripts putatively related to the *Dmrt* family of genes. The seven putative genes (dsx- and mab-3-related transcription factors) showed high similarity in the DM domain to known *Dmrt* genes in the GenBank database. The seven *MroDmrt* genes are 825–2565 nucleotides long, with predicted ORFs encoding 275–855 amino acid (aa) translation products. Two of the above genes, 2073 and 906 bp in length, which encode 537 and 205 aa proteins, respectively, were reported previously and termed, at that time, *MroDmrt11E* and *MroDmrt99B* (Yu et al., 2014). Four of the *Dmrt* genes, newly found in the present study, had high similarity to *SveiDmrt1*

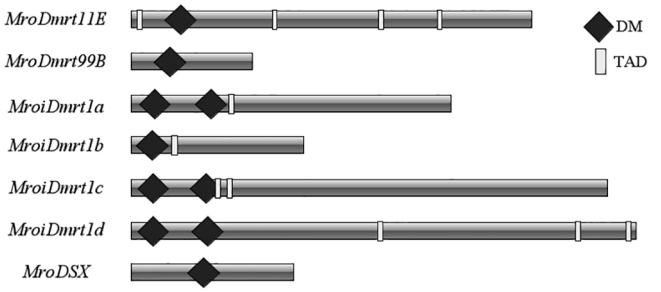

Fig. 1. Scaled illustration of the domain architecture of seven MroDMRT proteins. DM-domains are represented by diamonds and the predicted transactivation domains (TADs), by rectangles.

(Chandler et al., 2017), and were therefore named according to the degree of their similarity, *MroiDmrt1a-d* (accession numbers MK468649, MK468650, MK468651 and MK468652), respectively). The remaining gene was termed *MroDSX*- (accession number MK468653)) due to its high similarity to known decapod *DSX* genes (*FcDSX* and SveDSX) (Chandler et al., 2016; Li et al., 2018).

The cDNA and predicted protein sequences of the five newly described *Dmrt* genes are shown in the Supplementary material (Fig. S1a-e). Examination of the deduced amino acid sequences of all the putative *Dmrt* candidate genes revealed the conserved domains that characterize the Dmrt proteins reported to date. Specifically, each putative Dmrt included one or two DM domains. In addition, five of the predicted Dmrt proteins contained 1–4 TAD prediction domains (Fig. 1).

The phylogenic analysis confirmed that MroDmrt proteins do indeed cluster with similar Dmrt proteins from other species and even from remote taxa (Fig. 2).

3.2. Embryonic expression patterns

The expression of *MroDmrt* reads was studied in males and females at five embryonic stages. *MroDmrt* genes did not show any sex-specific expression pattern, but some of the genes were found to exhibit time-dependent expression patterns along embryonic development. These patterns were found to be similar in males and females, as may be seen in Fig. 3 for four representative genes. The expression of those genes was low at early embryonic stages and started to rise in later stages. For example, *MroiDmrt1b* started to rise on day 3, *MroDmrt11E* started to rise on day 5, whereas *MroDmrt99B* and *MroiDmrt1c* started to rise on day 11.

3.3. Larval and post-larval expression patterns

To study gene expression patterns during early developmental stages, the numbers of *DMRT*-reads per million (RPKM) per sample were mined from transcriptomic libraries of male larvae, female larvae, male PLs, and female PLs (Ventura et al., 2013) (Fig. 4). *MroiDmrt1c* and *MroiDmrt1d* had high RPKMs in the larval stage compared to the other putative *Dmrt* genes. The reads of *MroiDmrt1c* and *MroiDmrt1d* were even higher in the PL stage, in both males and females. For *MroiDmrt1c* there were about 120 RPKMs in the larval stage, and more than twofold that number in PLs of both genders. *MroiDmrt1d* exhibited 60–90 RPKMs in the larval stage, and more than fourfold that number in PLs of both genders. The remaining five *Dmrt* genes exhibited basal to low RPKMs (between 0 and 26) in both larval and PL stages.

3.4. Adult expression patterns

In adult males, the expression levels of the above *MroDMRT* genes were studied in an AG transcriptomic library that included the three male morphotypes—BC, OC and SM (Fig. 5a). *MroiDmrt1b* and *MroiDmrt1c* were found to be highly expressed in the AG. To study the

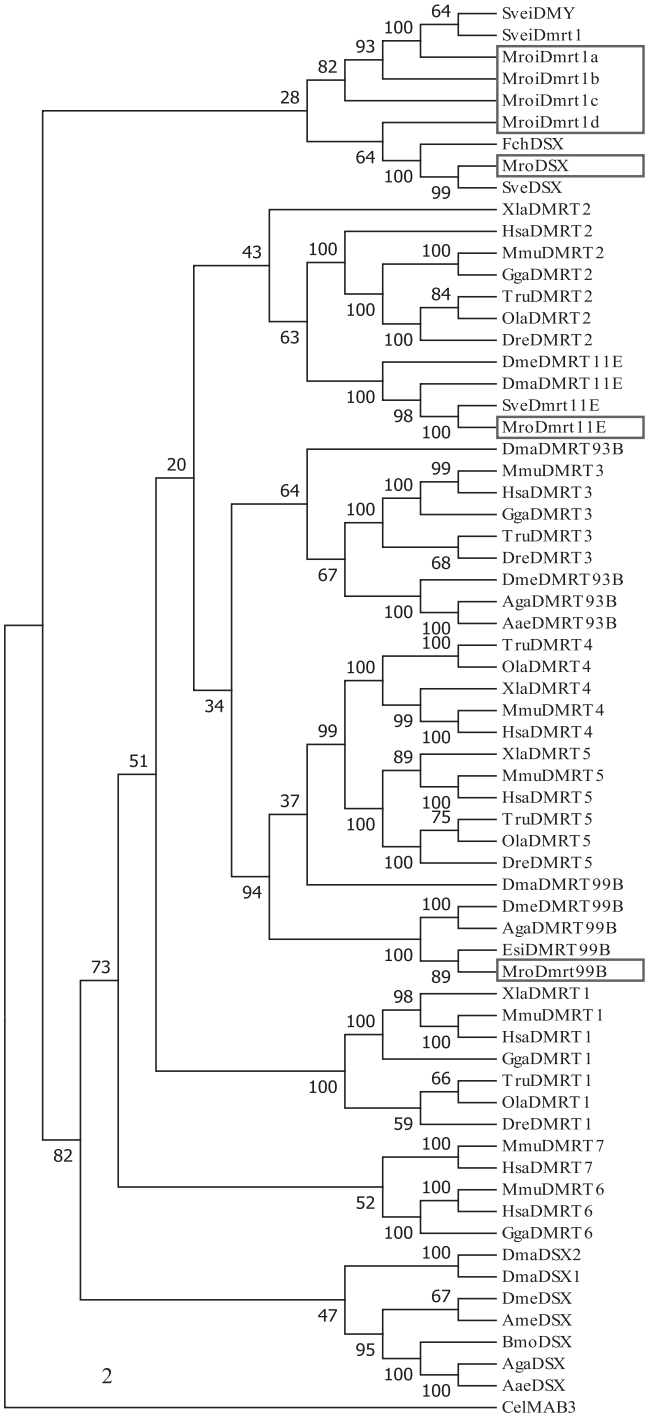

Fig. 2. Phylogenetic analysis based on multiple alignments of DMRT proteins. The neighbor-joining tree was constructed according to multiple alignments of DMRT entire amino acid sequences. The tree scale indicates the evolutionary distance in terms of the branch lengths, which are proportional to sequence differences. Bootstrap values are represented by the numbers alongside the branches. The list of proteins used for the construction of the phylogenetic tree is presented in Table 1.

specificity of this phenomenon to the AG, the expression of *MroiDmrt1b* and *MroiDmrt1c* in AGs was compared to that in eyestalks for the three male morphotypes. *MroiDmrt1b* and *MroiDmrt1c* were found to be significantly specifically enriched in the AG (Fig. 5b-c). The results for the other five genes, which were not specifically enriched in the AG as compared to the eyestalk, are given in the Supplementary material (Fig. S2a, d-g). In addition, further comparisons of *MroiDmrt1a*, *MroiDmrt1b*,

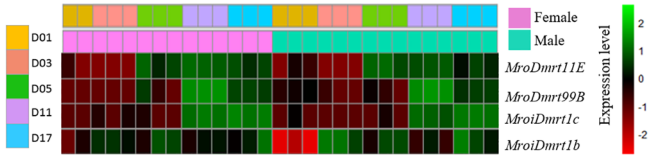

Fig. 3. Time-dependent expression of four representative *MroDmrt* genes, *MroDmrt11E*, *MroDmrt99B*, *MroiDmrt1c* and *MroiDmrt1b* during embryonic stages in males and females. The figure shows values for three replicates per day.

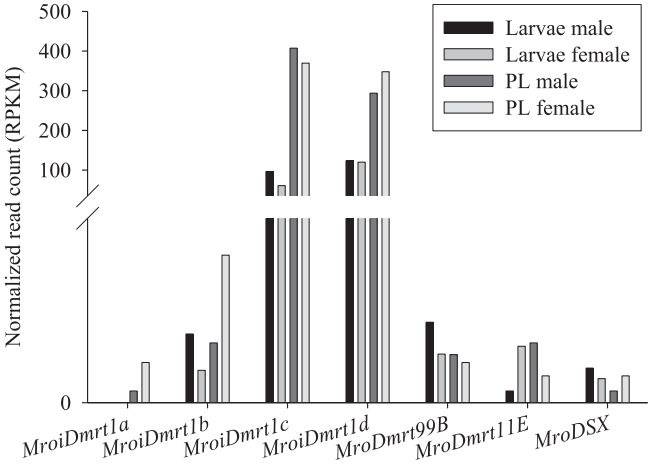

Fig. 4. Normalized read counts per million (RPKM) of *DMRT* genes originating from *M. rosenbergii* larvae and post larvae transcriptomic libraries from Ventura et al. (2013). Columns represent the two developmental stages (larvae and post-larvae) in males and females. The seven *DMRT* genes are represented on the X axis.

MroiDmrt1c and *MroDmrt11E* expression levels among ovary, testis and AG revealed different patterns (Fig. 6). *MroiDmrt1a* was significantly different among the tissues with the highest expression in the AG and the lowest in the ovary. *MroiDmrt1b* was highly expressed in the AG and testis and significantly lower in the ovary while contrary, *MroiDmrt1c* was highly expressed in the ovary and significantly lower in the AG and testis. *MroDmrt11E* was significantly higher in the testis compared to the ovary.

3.5. Mr-IAG silencing effects Dmrt gene expression

The two *MroDmrt* genes that were found to be enriched in the AG were further studied with the aim to reveal a possible relationship with the *IAG*-switch pathway. Specific silencing of *Mr-IAG* through administration of ds*Mr-IAG* showed a significant decrease in *Mr-IAG* expression ($P < 0.05$), with a silencing efficiency of 99.8% (Fig. 7a). The silencing of *Mr-IAG* significantly decreased the expression levels of *MoriDmrt1b* and *MroiDmrt1c*, i.e., by 95.4 and 71.9% in the *IAG* silenced group compared to the control, respectively ($P < 0.05$) (Fig. 7b&c). *Mr-IAG* silencing did not significantly affect the expression of the control genes, *Mr-actin* and *MroiDmrt1d* (Fig. 7d&e).

4. Discussion

The identification and characterization of sex-specific genes are essential steps to providing insight into the molecular pathways of sex determination and differentiation in crustaceans. In this study, we

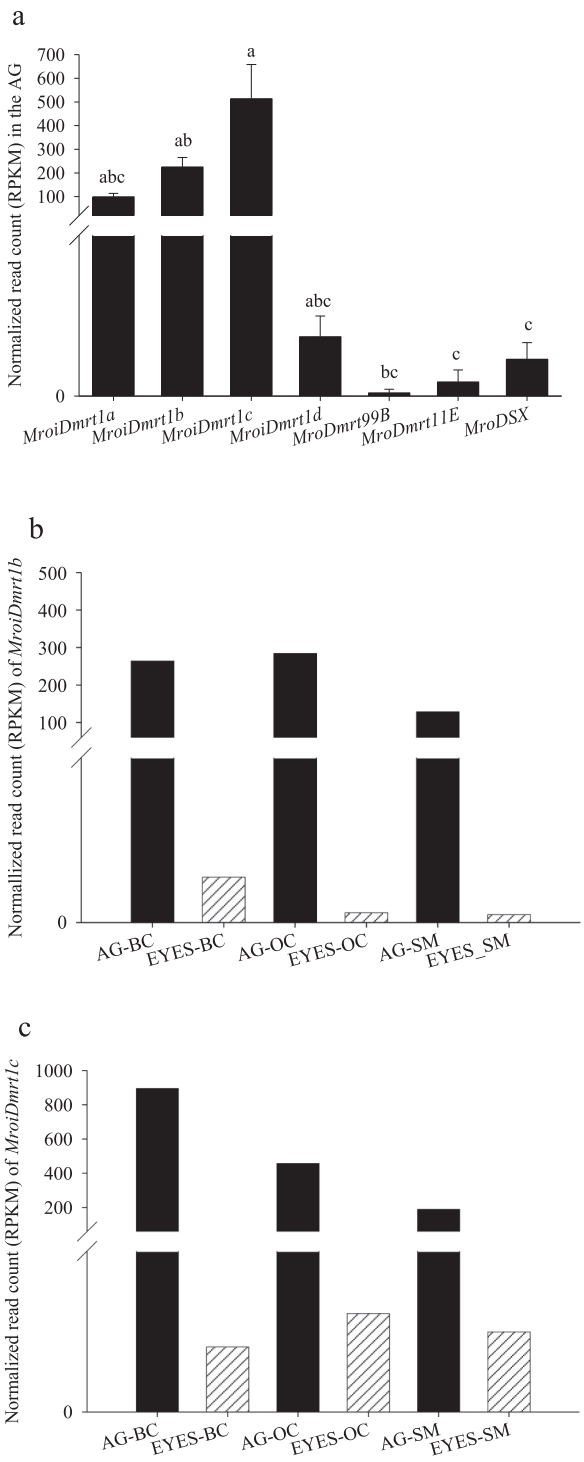

Fig. 5. Normalized read counts per million (RPKM) of *DMRT* genes originating from *M. rosenbergii* androgenic gland transcriptomic libraries, including the three male morphotypes, blue claw (BC), orange claw (OC) and small male (SM) from Sharabi et al. (2016). (a) Read counts of the seven *MroDMRT* genes in the androgenic gland. Different letters on the bars indicate significant differences ($P < 0.05$; post hoc Tukey's test). (b, c) Read counts of *MroiDmrt1b* and *MroiDmrt1c*, respectively, in the androgenic glands of the three male morphotypes as compared to the read counts of these genes in the eyestalks.

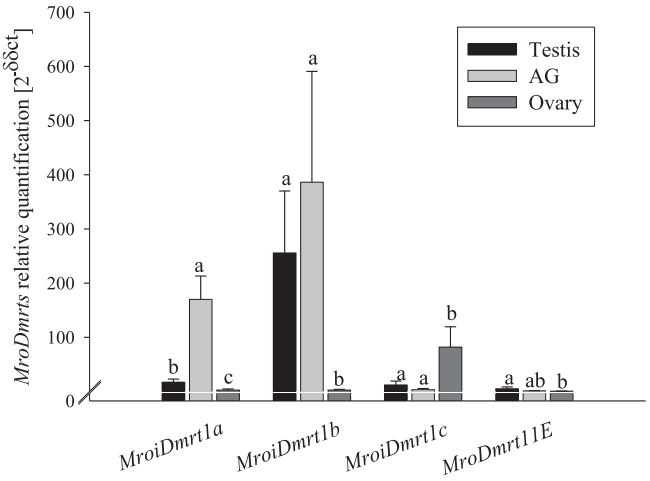

Fig. 6. Relative quantification of *MroDmrt1a*, *MroDmrt1b*, *MroDmrt1c* and *MroDmrt11E* in the gonads and in the AG from five females and six males. Different letters on the bars indicate significant differences ($P < 0.05$; post hoc Tukey's test) after natural log transformation of the data as to fit proper statistical analysis.

undertook a developmentally wide screening for *Dmrt* gene family members of transcriptomic libraries from embryo to adult *M. rosenbergii*. Our screen confirmed the presence of two known *Dmrt* family members, *MroDmrt11E* and *MroDmrt99B* (Yu et al., 2014), and identified five additional novel *Dmrt* genes in the prawn. The new transcripts were named according to their phylogenic homology to the known DMRT proteins; MroiDmrt1a, MroiDmrt1b, MroiDmrt1c, MroiDmrt1d, and MroDSX, encoded by 1623, 876, 2421, 2564, and 825 bp mRNA, respectively. MroDmrt proteins show a high similarity to known Dmrt proteins in the conserved DM domain, which consists of cysteines and histidines, forming two zinc finger binding sites that are essential for DNA binding (Zhu et al., 2000). *MroiDmrt1a and MroiDmrt1b* are predicted to contain one TAD, and *MroiDmrt1c*, *MroiDmrt1d* and *MroDmrt11E* are predicted to contain two, three and four TADs, respectively, where the TAD is a conserved domain in most known transcriptionally active Dmrts (Chandler et al., 2017), thus supporting a possible function of *MroDmrt* genes as transcription factors.

The *Dmrt* gene family has retained a conserved role in the sexual development of metazoans across evolution. For example, it is believed that the testis-specific *Dmrt* gene designated *EsDmrt*-like plays an important role in the reproductive system, particularly in testicular development, in the crab *E. sinensis*, since RNAi silencing of *EsDmrt*-like reduced testicular size and blocked spermatogenesis (Ma et al., 2016). Also important for male differentiation, the *Sv-iDMY* gene, the first heterogametic sex-specific *Dmrt* to be identified in an invertebrate species (Chandler et al., 2017), was suggested to play a dominant role over *Sv-iDmrt1*: Only in the absence of *Sv-iDMY*, is *Sv-iDmrt1* predicted to promote female development (Chandler et al., 2017). In another crustacean, *Daphnia magna*, three *Dmrt* genes (*DMRT11E*, *DMRT93B* and *DMRT99B*) were expressed dimorphically, with *DMRT11E* and *DMRT99B* expression levels being higher in the ovary than in the testis, and *DMRT93B* being expressed only in the testis (Kato et al., 2008). Unlike the above-mentioned cases, the *MroDmrt* genes found in the present study did not show any sex-specific expression pattern in either male or female embryos, but they were found to exhibit time-dependent expression patterns along embryonic development. However, in mature prawns, four *MroDmrt* genes exhibited distinct sexual dimorphic expression pattern among the gonads and the AG. These patterns might shed light on the roles of *MroDmrts* in gonads development. *MroiDmrt1b* was found to be expressed only in the testis and the AG and not in the ovary, whereas *MroiDmrt1a* was high in the testis and the AG compared to the ovary, suggesting possible role of these DM genes in gonad development in males depending on gene expression level. This phenomena was also demonstrated in birds, in which, *DMRT1* is expressed higher in males than females, specifically in the gonads of chicken embryos, before to and during gonadal differentiation (Smith et al., 2009; Smith and Sinclair, 2004). *MroDmrt11E* was highly expressed in the testis and the AG compared to the ovary, confirming previous results (Yu et al., 2014). *MroiDmrt1c*, unlike the above genes, was highly expressed in the ovary. However, it was expressed also in the testis and the AG, suggesting its possible role in both male and female gonadal development.

Even though no sex-specific expression pattern was found for *MroDmrt* genes in embryos, the silencing of a sex-related gene, the *IAG*, significantly reduced the expression of two genes, *MroiDmrt1b* and *MroiDmrt1c*, thereby suggesting their possible roles in the IAG-switch and the sex-differentiation mechanism. In light of the findings of a recent RNAi-targeting study (Yu et al., 2014), it should be stressed that these two genes have predicted TADs: in that study it was shown that RNAi-targeting of *MroDmrt99B*, which has no predicted TAD, had no effect on several male reproduction-related genes, while *MroDmrt11E* RNAi induced a significant decrease of the transcript of *IAG*. These findings suggest possible transcriptional activity of the TAD in MroDmrt11E. However, although previous studies have shown that *Dmrt* genes are regulating the *IAG* in some species (Li et al., 2018; Wang et al., 2019; Yu et al., 2014), in the present study we suggested that *MroiDmrt1b* and *MroiDmrt1c* are correlated with the *Mr-IAG* system but it is hard to conclude whether the relationship is up or downstream. Our efforts did not succeed in achieving loss of function of *Dmrt* genes through RNAi, and previous studies claiming successful loss of function are reporting conflicting results in which in *M. rosenbergii* (Yu et al., 2014) and *Fenneropenaeus chinensis* (Li et al., 2018) a reduction of expression of *IAG* was reported following Dmrt silencing while contrary to this study, in *Macrobrachium* nipponense (Wang et al., 2019) the opposite was shown. Thus, we cannot offer a clear conclusion regarding the regulatory role of *Dmrt* genes with respect to IAG expression.

Previous studies have indicated that *Dmrt* genes also belong to a family of important developmental regulators, providing evidence that this gene family has evolved functionally in relation to developmental pathways that are distinct from of sex-determination or sex-differentiation pathways (Hong et al., 2007). One example of such a non-sex related gene is *terra*, which encodes a putative zinc-finger (DM domain) protein in the zebrafish and is specifically expressed in the prismatic mesoderm and developing somite. It has been proposed that *terra* is a conserved somite-specific factor that mediates very early events of vertebrate somitogenesis (Meng et al., 1999). Many *Dmrt* genes that are expressed in the developing gonad appear to share similar functional characteristics. However, in non-gonadal tissues, Dmrt factors appear to have the ability to regulate a broad range of developmental processes (Hong et al., 2007), to which only limited attention has been paid to date. The time-dependent expression pattern of *MroDmrt* genes along the embryo, larval, post-larval and adult stages might suggest possible involvement of these proteins in somitogenesis rather than in reproductive development.

In light of our findings, it may be suggested that *Dmrt* genes play regulatory roles in both sexual and somatic development: On the one hand, in *M. rosenbergii* the expression pattern of *Dmrt* genes was similar in male and female embryos, while on the other hand, some of the genes show different expression patterns among gonads in mature males and females. In addition, silencing of *IAG*, a pivotal gene in sexual development, caused a significant decrease in the expression of two *Dmrt* genes. Further studies are thus needed to determine the precise function of *Dmrt* genes in the developing reproductive system and other tissues.

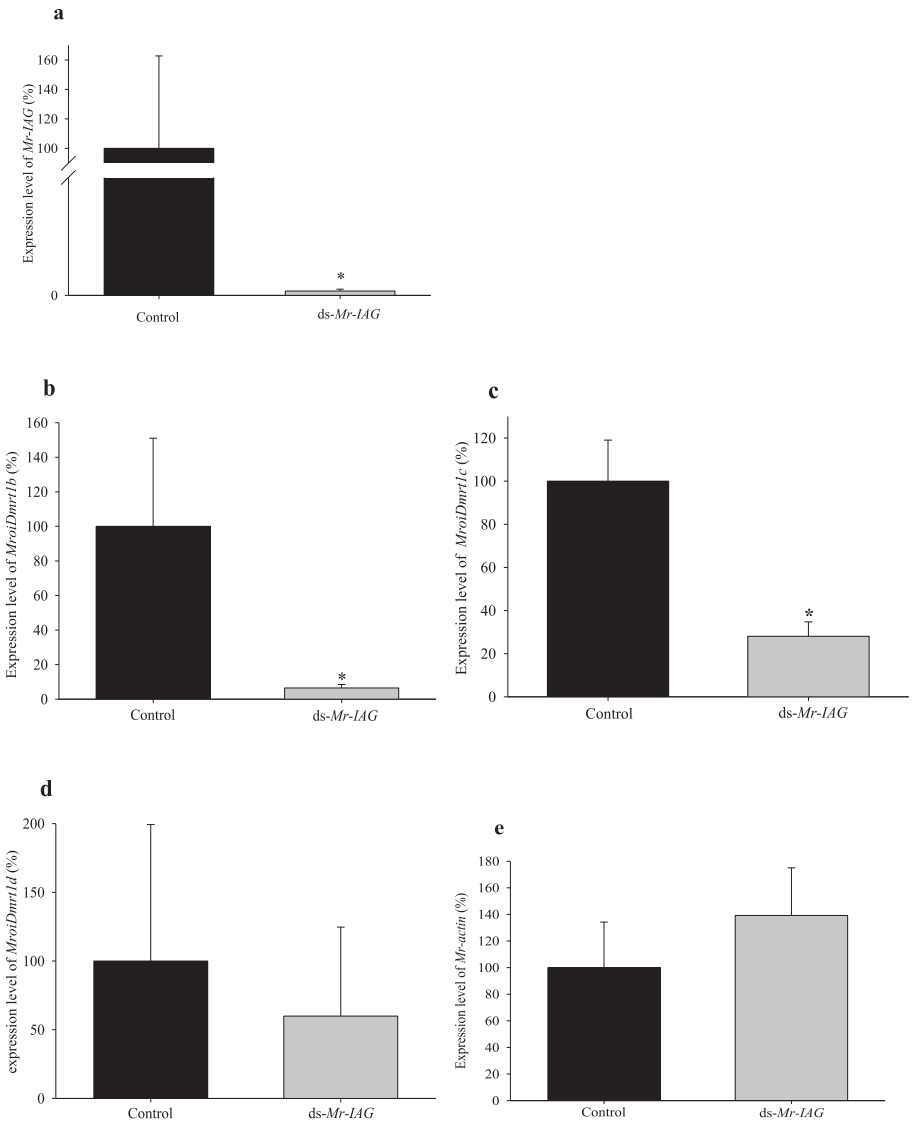

Fig. 7. Levels of transcripts in the androgenic gland following *in vivo Mr-IAG* dsRNA injection into adult male prawns. Levels of (a) *Mr-IAG* transcripts; (b&c) *MroiDmrt1b and MroiDmrt1c* transcripts, respectively, and (d&e) control genes, *Mroidmrt1d* and *Mr-actin*, respectively. Asterisk indicates significant difference ($P < 0.05$; Mann Whitney *U* test).

Acknowledgements

We thank Dr. Vered Chalifa-Caspi and Dr. Menachem Sklarz for their help with the bioinformatics analyses, and the Dor Research Center for housing and maintaining the experimental prawns. This study was supported in part by the Israel Science Foundation (ISF) within the ISF-UGC joint research program (grant No. 2728/16).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.06.009.

References

Chandler, J.C., Aizen, J., Elizur, A., Battaglene, S.C., Ventura, T., 2016. Male sexual development and the androgenic gland: novel insights through the de novo assembled transcriptome of the eastern spiny lobster, *Sagmariasus verreauxi*. Sex. Dev.

Chandler, J.C., Fitzgibbon, Q.P., Smith, G., Elizur, A., Ventura, T., 2017. Y-linked iDmrt1 paralogue (iDMY) in the Eastern spiny lobster, *Sagmariasus verreauxi*: the first invertebrate sex-linked Dmrt. Dev. Biol. 430, 337–345.

Charniaux-Cotton, H., 1954. Discovery in, an amphipod crustacean (*Orchestia gammarella*) of an endocrine gland responsible for the differentiation of primary and secondary male sex characteristics. C. R. Hebd. Seances Acad. Sci. 239, 780–782.

Chen, Y., Fan, H., Hsieh, S., Kuo, C., 2003. Physiological involvement of DA in ovarian development of the freshwater giant prawn, *Macrobrachium rosenbergii*. Aquaculture 228, 383–395.

Hildreth, P.E., 1965. *Doublesex*, a recessive gene that tranforms both males and females of *Drosophila* into intersexes. Genetics 51, 659.

Hodgkin, J., 2002. Exploring the envelope: systematic alteration in the sex-determination system of the nematode *Caenorhabditis elegans*. Genetics 162, 767–780.

Hong, C.-S., Park, B.-Y., Saint-Jeannet, J.-P., 2007. The function of Dmrt genes in vertebrate development: it is not just about sex. Dev. Biol. 310, 1–9.

Jung, H., Yoon, B.-H., Kim, W.-J., Kim, D.-W., Hurwood, D.A., Lyons, R.E., et al., 2016. Optimizing hybrid de novo transcriptome assembly and extending genomic resources for giant freshwater prawns (*macrobrachium rosenbergii*): The identification of genes and markers associated with reproduction. Int. J. Mol. Sci. 17, 690.

Karplus, I., Sagi, A., 2000. The biology and management of size variation. New York, 259–289.

Kato, Y., Kobayashi, K., Oda, S., Colbourn, J.K., Tatarazako, N., Watanabe, H., et al., 2008. Molecular cloning and sexually dimorphic expression of DM-domain genes in *Daphnia magna*. Genomics 91, 94–101.

Kato, Y., Kobayashi, K., Watanabe, H., Iguchi, T., 2011. Environmental sex determination in the branchiopod crustacean *Daphnia magna*: deep conservation of a doublesex gene in the sex-determining pathway. PLoS Genet. 7.

Khalaila, I., Katz, T., Abdu, U., Yehezkel, G., Sagi, A., 2001. Effects of implantation of hypertrophied androgenic glands on sexual characters and physiology of the reproductive system in the female red claw crayfish, *Cherax quadricarinatus*. Gen. Comp. Endocr. 121, 242–249.

Kopp, A., 2012. Dmrt genes in the development and evolution of sexual dimorphism.

Trends Genet. 28, 175–184.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
Kuris, A.M., Raanan, Z., Sagi, A., Cohen, D., 1987. Morphotypic differentiation of male Malaysian giant prawns, *Macrobrachium-rosenbergii*. J. Crustacean Biol. 7, 219–237.
Larkin, M.A., Blackshields, G., Brown, N., Chenna, R., McGettigan, P.A., McWilliam, H., et al., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.
Levy, T., Rosen, O., Eilam, B., Azulay, D., Aflalo, E.D., Manor, R., et al., 2016. A single injection of hypertrophied androgenic gland cells produces all-female aquaculture. Mar. Biotechnol. 18, 554–563.
Li, S., Li, F., Yu, K., Xiang, J., 2018. Identification and characterization of a doublesex gene which regulates the expression of insulin-like androgenic gland hormone in *Fenneropenaeus chinensis*. Gene.
Ma, K.-Y., Chen, J., Liu, Z.-Q., Qiu, G.-F., 2016. Inhibitory effects of RNAi-mediated knockdown of *EsDmrt-like* gene on testicular development in the Chinese mitten crab *Eriocheir sinensis*. Aquaculture 463, 217–223.
Ma, W.-J., Vavre, F., Beukeboom, L.W., 2014. Manipulation of arthropod sex determination by endosymbionts: diversity and molecular mechanisms. Sex. Dev. 8, 59–73.
Malecha, S.R., Nevin, P.A., Ha, P., Barck, L.E., Lamadridrose, Y., Masuno, S., et al., 1992. Sex-ratios and sex-determination in progeny from crosses of surgically sex-reversed freshwater prawns, *Macrobrachium rosenbergii*. Aquaculture 105, 201–218.
Manush, S., Pal, A., Chatterjee, N., Das, T., Mukherjee, S., 2004. Thermal tolerance and oxygen consumption of *Macrobrachium rosenbergii* acclimated to three temperatures. J. Therm. Biol. 29, 15–19.
Mapp, A.K., Ansari, A.Z., 2007. A TAD further: exogenous control of gene activation. ACS Chem. Biol. 2, 62–75.
Meng, A., Moore, B., Tang, H., Yuan, B., Lin, S., 1999. A *Drosophila doublesex*-related gene, *terra*, is involved in somitogenesis in vertebrates. Development 126, 1259–1268.
Miller, S.W., Hayward, D.C., Bunch, T.A., Miller, D.J., Ball, E.E., Bardwell, V.J., et al., 2003. A DM domain protein from a coral, *Acropora millepora*, homologous to proteins important for sex determination. Evolution & Development 5, 251–258.
Nair, C.M., Salin, K.R., Raju, M.S., Sebastian, M., 2006. Economic analysis of monosex culture of giant freshwater prawn (*Macrobrachium rosenbergii* De Man): a case study. Aquac Res. 37, 949–954.
New, M.B., 2008. Freshwater Prawn Culture: The Farming of *Macrobrachium rosenbergii*. John Wiley & Sons.
Okumura, T., Hara, M., 2004. Androgenic gland cell structure and spermatogenesis during the molt cycle and correlation to morphotypic differentiation in the giant freshwater prawn, *Macrobrachium rosenbergii*. Zool. Sci. 21, 621–628.
Raymond, C.S., Kettlewell, J.R., Hirsch, B., Bardwell, V.J., Zarkower, D., 1999. Expression of *Dmrt1* in the genital ridge of mouse and chicken embryos suggests a role in vertebrate sexual development. Dev. Biol. 215, 208–220.
Raymond, C.S., Shamu, C.E., Shen, M.M., Seifert, K.J., Hirsch, B., Hodgkin, J., et al., 1998. Evidence for evolutionary conservation of sex-determining genes. Nature 391, 691.
Roth, Z., Weil, S., Aflalo, E.D., Manor, R., Sagi, A., Khalaila, I., 2013. Identification of receptor-interacting regions of vitellogenin within evolutionarily conserved β-sheet structures by using a peptide array. Chembiochem. 14, 1116–1122.
Sagi, A., Cohen, D., 1990. Growth, maturation and progeny of sex-reversed *Macrobrachium rosenbergii* males. World Aquacult. 21, 87–90.
Sagi, A., Cohen, D., Milner, Y., 1990. Effect of androgenic gland ablation on morphotypic differentiation and sexual characteristics of male fresh-water prawns, *Macrobrachium rosenbergii*. Gen. Comp. Endocr. 77, 15–22.
Schultz, J., Milpetz, F., Bork, P., Ponting, C.P., 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. 95, 5857–5864.
Sharabi, O., Manor, R., Weil, S., Aflalo, E.D., Lezer, Y., Levy, T., et al., 2016. Identification and characterization of an insulin-like receptor involved in crustacean reproduction. Endocrinology 157, 928–941.
Shpak, N., Manor, R., Aflalo, E.D., Sagi, A., 2017. Three generations of cultured prawn without W chromosome. Aquaculture 467, 41–48.
Sklarz, M.Y., Levin, L., Gordon, M., Chalifa-Caspi, V., 2017. NeatSeq-Flow: A Lightweight High Throughput Sequencing Workflow Platform for Non-Programmers and Programmers alike.
Smith, C.A., Roeszler, K.N., Ohnesorg, T., Cummins, D.M., Farlie, P.G., Doran, T.J., et al., 2009. The avian Z-linked gene *DMRT1* is required for male sex determination in the chicken. Nature 461, 267.
Smith, C.A., Sinclair, A.H., 2004. Sex determination: insights from the chicken. Bioessays 26, 120–132.
Veenstra, J.A., 2016. Similarities between decapod and insect neuropeptidomes. PeerJ. 4, e2043.
Ventura, T., Manor, R., Aflalo, E.D., Chalifa-Caspi, V., Weil, S., Sharabi, O., et al., 2013. Post-embryonic transcriptomes of the prawn *Macrobrachium rosenbergii:* multigenic succession through metamorphosis. Plos One 8.
Ventura, T., Manor, R., Aflalo, E.D., Weil, S., Rosen, O., Sagi, A., 2012. Timing sexual differentiation: full functional sex reversal achieved through silencing of a single insulin-like gene in the prawn, *Macrobrachium rosenbergii*. Biol. Reprod. 86, 1–6.
Wang, Y., Jin, S., Fu, H., Qiao, H., Sun, S., Zhang, W., et al., 2019. Identification and characterization of the *DMRT11E* gene in the oriental river prawn *Macrobrachium nipponense*. Int. J. Mol. Sci. 20, 1734.
Yu, Y.-Q., Ma, W.-M., Zeng, Q.-G., Qian, Y.-Q., Yang, J.-S., Yang, W.-J., 2014. Molecular cloning and sexually dimorphic expression of two Dmrt genes in the giant freshwater prawn, Macrobrachium rosenbergii. Agric. Res. 3, 181–191.
Zhang, E.F., Qiu, G.F., 2010. A novel Dmrt gene is specifically expressed in the testis of Chinese mitten crab, *Eriocheir sinensis*. Dev. Genes Evol. 220, 151–159.
Zhu, L., Wilken, J., Phillips, N.B., Narendra, U., Chan, G., Stratton, S.M., et al., 2000. Sexual dimorphism in diverse metazoans is regulated by a novel class of intertwined zinc fingers. Genes & Development 14, 1750–1764.
Zhu, X.-J., Xiong, Y., He, W., Jin, Y., Qian, Y., Liu, J., et al., 2018. Molecular cloning and expression analysis of a prawn (*Macrobrachium rosenbergii*) juvenile hormone esterase-like carboxylesterase following immune challenge. Fish Shellfish Immunol.